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14. ABSTRACT

Breast cancer is a complex disease that develops from epithelial lesions confined to breast ducts and lobules and progresses rapidly to become locally invasive and finally metastatic. Our recent studies show that breast cancer cells undergo asymmetric events during cell division that generate different daughter cells. One daughter receives the singular midbody (MB) that is made during every cell division. The cell with this so-called postmitotic *midbody derivative* accumulates additional MBds with successive divisions. In breast tumor sections, rare cells stain for MBds adjacent to the basal layer, the position of putative breast cancer stem cells. MBds are present in high numbers in several human breast cancer cell lines and in human tumors, but are rarely found in normal breast epithelial cell lines or breast tissue. MBds are also found in several well-characterized mouse and human stem cell niches but not in adjacent transit amplifying or differentiating cells. These results suggest that MBds are in almost exclusively in stem cells and putative breast cancer stem cells (CSS). This idea is consistent with the emerging view that breast cancer develops from transformation of stem cells.

Based on these observations, we propose that MBds 1) will serve as markers for breast CSCs, 2) may have diagnostic/prognositc value for breast cancer progression and 3) could directly contribute to breast carcinoma. To test this, we propose the following aims: 1) Quantify MBds in breast tumors and cell lines and compare with normal breast epithelial cells. 2) Test MBd-containing breast cancer cells for CSC activities in vivo and in vitro. 3) Test MBds for their ability to confer breast cancer stem cell properties by disrupting MBd inheritance or RNAi.

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INTRODUCTION:

Breast cancer is a complex disease that develops from epithelial lesions confined to breast ducts and lobules and progresses rapidly to become locally invasive and finally metastatic. Our recent studies show that cancer cells undergo a series of asymmetric events during cell division that generate *different* daughter cells (Gromley et al, Cell, 2005). We have shown this is also the case for breast cancer cells (MDA-MB-231, MCF-10A CA1a). Remarkably, one daughter inherits the midbody (MB), an organelle that assembles between the two dividing daughter cells. We call this post-division MB, the *midbody derivative (MBd).* Remarkably, the cell with the MBd accumulates additional MBds with each subsequent cell division. In human breast tumor sections, we observed rare cells adjacent to the basal layer--the position of putative breast cancer stem cells (CSCs)--that stain for MBds. In the breast cancer cell line MDA-MB-231 MBds were present in high numbers. High MBd numbers were also found in several stem cell compartments and in human embryonic stem cells (hESCs, H9, iPS), and they dramatically decreased when stem cells were induced to differentiate. MBds were *not* detected in normal and near normal breast epithelial cells (e.g. HMECs, MCF10A), dividing hepatocytes in regenerating liver, activated T cells or transit amplifying cells in all tissues studied. These results suggest that MBds are present in normal SCs and breast CSCs in vivo and in vitro. Our work is consistent with the emerging view that breast cancer develops from transformation of stem cells (Dontu et al, 2005; Ponti et al, 2005). We propose that MBds 1) will serve as markers for breast CSCs, 2) may have diagnostic/prognostic value for breast cancer progression and 3) could directly contribute to breast carcinoma. To test this, we propose the following aims: 1) Quantify MBds in breast tumors and cell lines and compare them with normal breast epithelial cells. 2) Test MBd-containing breast cancer cells for CSC properties in vivo and in vitro. 3) Test MBds for the ability to confer breast cancer stem cell properties by disrupting MBd inheritance or by RNAi.

BODY:

We have made significant progress since initiating work on this proposal. The major accomplishments stem from work on Tasks 1 and 2 of the proposal. We have not yet initiated experiments described in Task 3, in keeping with the timeline of the proposal (Task 3 will initiate 12-18 months after the start of the grant).

For Task 1 we have quantified MBds in several breast cancer cell lines. We used MKLP1 as marker to detect both mitotic MBs and postmitotic MBds. To discern between midbodies and midbody derivatives, we used Aurora B kinase, which stains MBs but is lost when they are transferred into cells to become MBds. We considered cells with more than one MBd as evidence for ‘accumulation’, as even normal cells will have one MBd if it had just completed cell division and transferred the mitotic MB into the cell. We quantify the percentage of % MBd+ cells/total cells in the population. Figure 1 shows a representative image of an MDA-MB-231cell with multiple MBds stained with our main MBd marker, MKLP1 (from original proposal). The graph in Figure 2 (lower panel), generated from work done during this work period, shows the percent of breast cancer

cells with >1 MBd for MCF7, MCF10AT, MCF10CA1a) as well as normal breast epithelial cells (MCF10A); other control and tumor cells are included for comparison. There are two important observations that should be noted. First, the percentage of MCF7 cells with >1 MBd is higher than all other cancer cells examined, including human embryonic stem cells. The significance of this observation is unclear, although we have begun to look at the tumorigenic potential of cell lines within this panel of tumor cells. Perhaps more interesting, are observations from a series of genetically matched cell lines derived from the normal breast epithelial cell line (MCF10A). One variant of this line (MCF10AT) is transformed with Ha-ras (MCF10AT) and forms premalignant lesions that progress to non-malignant cancers. A line derived from MCF10AT called MCF10CA1a, is fully malignant and metastatic. This series of cell lines defines steps in the breast cancer tumorigenic process from normal to metastatic tumor. The percentage of MBds in the MCF10A line is very low, as in all normal control cells. The percentage of MBds in MCF10AT is ten-fold higher than MCF10A. The percentage of MBds in MCF10CA1a is 1.6-fold higher than in MCF10AT (and thus 16-fold higher than MCF10A). These results show an excellent correlation between increased percentage of cells with MBds and increasing breast cancer malignancy.

A trivial explanation for the accumulation of MBds in breast cancer cells versus matched normal breast epithelial cells is that cell division in breast cancer cells is faster than normal breast epithelial cells, thus preventing MBds from being degraded before the next division. Thus, rapidly dividing cells would accumulate MBds while slow growing cells would have time to degrade them, making them devoid of MBds when entering the next division cycle. We examined this hypothesis by determining the cell-doubling rate of the three breast cancer cell lines, the normal breast epithelial cell line and others for comparison. Doubling times were determined by cell counting on a hemocytometer or by colorimetric MTS-based proliferation assay, done in parallel. Figure 2 (upper panel) demonstrates that no correlation exists between MBd accumulation and proliferation rate. For example the MCF10A normal breast epithelial cells divide more rapidly than two of the three breast cancer cell lines. More specifically, MCF10A divided at about the same rate as the MCF10AT cancer cells even tough they had ten-fold less MBds. Moreover, the doubling rate of MCF10A was 30% higher than most aggressive cancer cell line in this series, MCF10CA1a even though MCF10CA1a had ~16-fold more MBds. MCF7 cells divide the slowest of these breast cancer cell lines. This suggests that MBd accumulation is not a consequence of cell division timing.

The above methods do not rule out another plausible mechanism for MBd accumulation. It is possible that the subpopulation of cells that accumulate MBds cycle faster than the remainder of the population. To test this, we performed EdU-mediated pulse-chase experiments (similar to BrdU) on MCF10AT cells. The expectation is that the EdU signal intensity would be approximately halved after each cell division. If MBd-accumulating cells cycled faster than the rest of the population, one would expect that the EdU staining intensity would decrease more rapidly than the rest of the population. The results in Figure 3 show that EdU intensity of MBd-accumulating MCF10AT cells was not significantly different from rest of the population demonstrating that MBd-accumulating cells did not progress through the cell cycle any faster than rest of the cells. Taken

together, our data demonstrate that there is no significant correlation between population doubling time (Fig. 2) or single cell cycling rates (Fig. 3) and the percentages of MBd-accumulating/retaining cells in a population. This also suggests that MBd accumulation is a cell type-specific trait.

We are currently analyzing MBds in cells isolated from human breast tumors and we have some exciting preliminary data on MBds in human breast tumor sections. In paraffin and frozen sections from 3 independent tumors we observed a dramatic increase (up to 30-fold) in the percentage of cells with MBds compared with adjacent nontumor tissue. We are confirming this with additional samples using both tissue sections and isolated cells. We are also isolating breast cancer 'stem cells' to test the percent of MBds in this population. It should be noted that Fig. 1 shows work from a number of non-breast cancer cell lines—this work is not supported by this proposal but is compiled from other studies supported by the WM Keck Foundation. These data help put the data from the breast cancer cells and breast epithelial cells in perspective.

For Task 2, we have generated an MCF7 cell line constitutively expressing GFP-MKLP1 to label and track MBds in living cells. Other lines are being made. One goal that we wanted to achieve with these cells was to use flow cytometry to enrich for MBd+ cell populations. However, this was not as successful as we hoped, and we obtained only a small enrichment of MBd+ cells (from 28 to 35% of cells with one MBd). We are optimizing this method using Amnis Imagestream technology. In the meantime, we found that we could isolate MBds using a micromanipulator. To accomplish this we simply trypsinized the GFP-MCF7 cells, identified GFP+ cells, and moved them to a separate area in the dish (50-200 cells collected/hour; the same approach was used for MB-negative cells).

While this strategy does not result in large cell numbers of cells, we obtained enough material of high purity (100% MBd+) to perform some soft agar assays and for tumor induction studies in mice using conventional methods. We are pleased to report that our preliminary data suggest that MBd+ cells performed better in both assays. MBd+ cells formed 4.7+/-2.1-fold more colonies in soft agar (n=2 experiments) showing that they are able to grow better in an anchorage dependent manner. MBd+ cells also established tumors in mice earlier than MBd-negative cells (2/6 vs. 0/4 initiate tumors in MBd+ vs. MBd-negative cells, respectively) and grew larger with time. We have injected a second and third set of mice and are awaiting results. We will also test cells with greater MBd number in tumor assays in vitro and in vivo. For these analyses, we will hand pick cells with 2 MBds or 3 MBds or more. However, cells with increasing numbers of MBds/cell are rare. We believe we may have found a way to increase cells with higher MBd numbers (below). We are aware the number of experiments and animals remains low thus far but the data are encouraging at this point.

We performed a simple experiment to test if MBds were degraded in cells (an alternative to the first model of cell division timing (above). As a simple test, we treated with inhibitors of lysosomal enzymes and found that the number of MBds per cell went up rather dramatically. Moreover, we found that degradation is through the autophagy

pathway (self eating) and found that a receptor for targeting some proteins for autophagic degradation is required for MBd degradation. Constitutive downregulation of this receptor using shRNA increases MBd from 1.5 on average to about 4/cell. We do not plan to pursue further the mechanism of autophagic degradation of MBds but we will continue to exploit this new mechanism for manipulating MBds to test whether cells with increased MBds/cell have more tumorigenic activity.

During the course of these studies we identified additional markers for MBds and these allow us more flexibility in performing immunofluorescence analyses using multiple antibodies or different fixation conditions. These include antibodies to MgcRacGAP and anillin.

KEY RESEARCH ACCOMPLISHMENTS:

* MBds are significantly higher in breast cancer cell lines than in normal breast epithelial cells
*MBd+ GFP-MKLP1-MCF7 cells isolated by micromanipulation form more colonies in soft agar than MB-negative cells
* Preliminary results indicate that MBd+ GFP-MKLP1-MCF7 cells establish tumors more rapidly and form larger subcutaneous tumors in mice.
*Discovered that nontumor cells (MCF10A) do not accumulate MBds because they degrade them in lysosomes through the autophagic pathway.
*Preliminary results indicate that MBds are present in human tumors but rarely in adjacent nontumor tissue (>30-fold increase).

REPORTABLE OUTCOMES:

* Constructed GFP-MKLP1- MCF7 cell lines
* Manuscript in preparation on MBds in breast cancer cells
* Dr. Doxsey was appointed Chair of the search committee for a Director of UMass Medical School's future Stem Cell and Regenerative Medicine Institute.
* I received and accepted several invitations to give seminars based on this work:

08/2008	Woods Hole, Massachusetts, *"Ellison Medical Foundation Meeting"*
09/2008	Jenna, Germany, *"Cell Biology of Mitosis Symposium"*
09/2008	Heidelberg, Germany, *"Spindle Poles and Centrosomes Symposium"*
10/2008	Athens, Georgia, University of Georgia
10/2008	Athens, Greece, *"Centrosome defects, mitosis, stem cells & cancer"*
12/2008	New York, NY, Sloan Kettering, *"Emerging Roles of Centrosomes"*.
01/2009	AstraZeneca
01/2009	Watertown, MA, Boson Biomedical Research Institute
01/2009	Ohio Sate University, Dept. Genetics
02/2009	ETH, Zurich, Switzerland
04/2009	A.I. DuPont Hosp. for Children, Willmington, DE
03/2009	NIH, Section on Structural Biology
07/2009	*Little People of America Meeting,* Brooklyn, NYC

CONCLUSION: In conclusion, we have made progress on the aims of our proposal and made an unexpected observation. We have shown that MBds are in a subset of cells in breast cancer cell lines and in breast tumors. Importantly, they are rarely seen in nontumor tissue and in normal, nontumor cell lines (e.g. MCF10A). Given their localization to stem cells in normal stem cell niches and in human embryonic stem cells, they may also be markers for breast cancer stem cells. This observation has important implications for the identification and isolation of breast cancer stem cells. What is most important to determine, is whether MBds *contribute to* stem cell activities. This work is in progress. We are beginning to target new pathways for eliminating MBds in breast cancer cells. Results on another proposed task looks promising as well. Preliminary results suggest that MBD+ cells have more tumorigenic potential than MBD- cells of the same cell line. This indicates that the MBd+ cells have stem cell like capabilities, in that they may generate rapidly dividing transit amplifying cells that differentiate (or not) into breast tumors. We are investigating this. A third effort is also moving forward. We made the surprising discovery that autophagy plays a role in MBd degradation. This provides us with a new tool to manipulate MBd numbers in cells and to test the role of these structures in stem cell self-renewal or differentiation. We will not pursue autophagy per se but rather exploit the pathway for MBd manipulation. Finally, we found that the number of MBd-containing cells in human tumors is dramatically higher than nontumor tissues. Thus, any progress on cell lines that shows promise will likely be more impressive in human tumor tissue. This also has important implications for targeting of MBds for therapeutic purposes, if our work brings us to this point.

REFERENCES:

A. Gromley et al, Cell 123, 75-87, 2005.
D. Ponti et al, Cancer Res. 5506-11, 2005.
G. Dontu et al, Stem Cell Rev, 207-13, 2005.

APPENDICES:

Figures and legends

SUPPORTING DATA: See appendix

Fig. 1

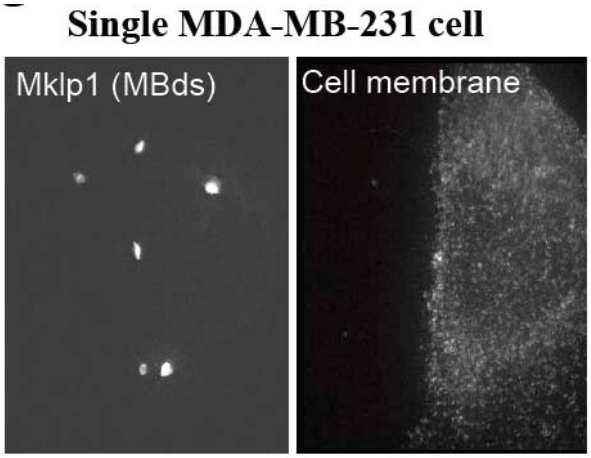

Fig. 2

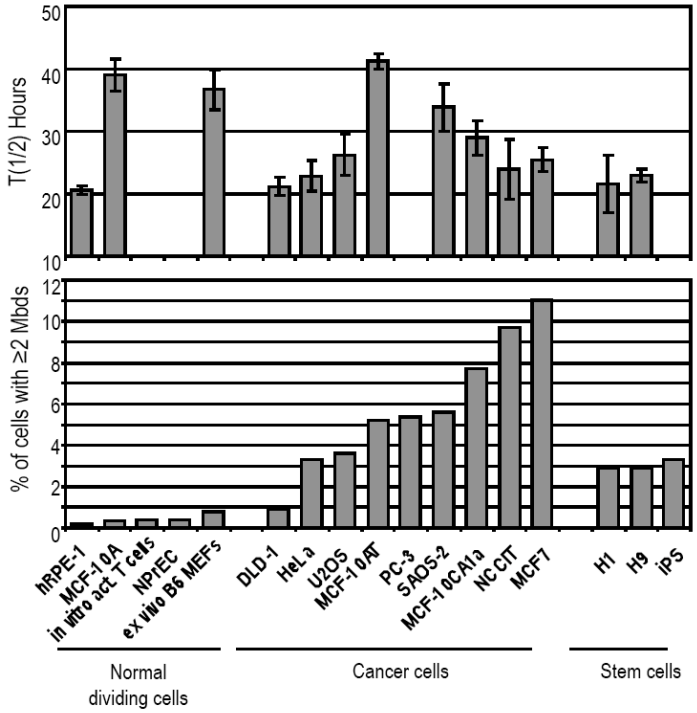

Fig 3

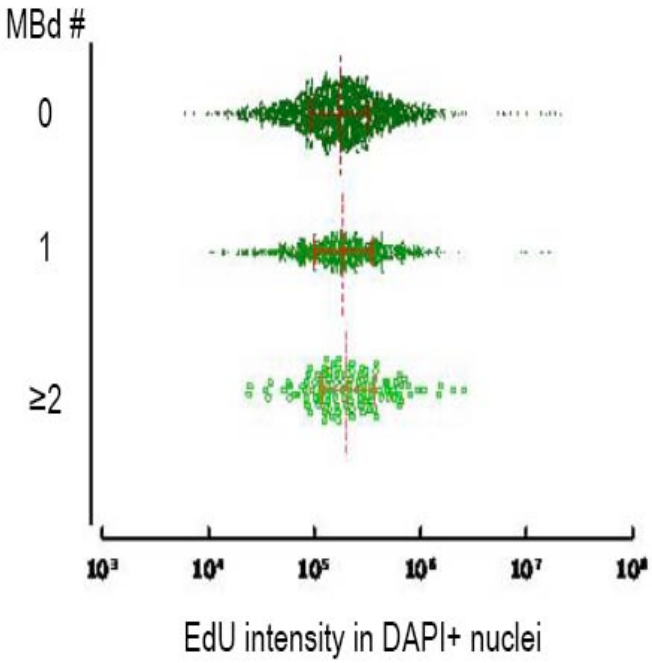

Figure legends. **Fig. 1** shows midbody derivatives (MBds) in an MDA-MB-231 cell stained with MKLP1 (left) and the cell membrane for reference (right). **Fig. 2** shows MBd numbers and cell cycle times of breast cancer cells (MCF10AT, MCF10CA1a) and breast epithelial cells (MCF10A). Bottom graph, percentage of cells with > or = 2 MBds. Note: normal breast epithelial cells (MCF10A, left section, 'normal') show a low percentage of cells with MBds (~0.5%), consistent with other controls (e.g. hRPE-1 cells, in vitro activated T cells, normal prostate epithelial cells [NPtEC] and ex vivo MEFs). In contrast, the breast cancer cell lines MCF10AT and MCF10CA1a show dramatically higher levels of cells with MBds (center section, "cancer") and the malignant breast cancer cells with MBds (MCF10CA1a) are higher than the less aggressive MCF10AT. Top graph, doubling times of MCF7, MCF10AT, MCF10CA1a and MCF10A. This, compiled with data from other cell lines, shows no correlation between doubling time and MBd accumulation. **Fig. 3.** EdU labeling after a 96 hour chase shows that individual MBd+ cells are not cycling faster than the MBd-negative cells. Each cell is represented by an individual dot on the graph. X axis, EdU labeling intensity of each nucleus plotted as logarithmic scale. Y-axis, number of MBds/cell. Cells counted: MBd-negative, n=1316; 1 MBd, n= 583; >1 MBd, n=109. Red dashed lines and the bars indicate the median and interquartile range of EdU intensity within each category. The distribution and the average EdU intensity among the three groups is not significantly different (p=0.2101, One-way ANOVA). Graph is representative of three experiments. DAPI, stains nuclei of all cells.